Supplementary Information

Fast volumetric calcium imaging across multiple cortical layers using sculpted light

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Supplementary Table 1 Comparison of s-TeFo to existing volumetric high-speed imaging techniques

Supplementary Note 1 Rational for using an enlarged sculpted excitation PSF and signal to noise in s-TeFo versus diffraction limited excitation

As stated in the main manuscript, one of the underlying ideas in our imaging approach is that for a given FOV and resolution, choosing the voxel size, i.e. the 3D size of the laser focus, to provide just the desired spatial resolution will result in the highest frame acquisition rate. This is because, provided no limitations in available laser power, biological damage or mechanical scan speed, the plane exposure time t_{exp} required to acquire the signal from the desired field-of-view, A_{FOV} depends on the excitation area A and its dwell time Δt , via $t_{exp} = (A_{FOV}/A) \cdot \Delta t$. Therefore, for a constant dwell time, therefore the largest possible value for A for a desired spatial resolution will result in the minimum number of voxels that need to be scanned for a given A_{FOV} and thus will result in the shortest plane exposure time t_{exp}.

The dwell time needs to be adequately long so that sufficient fluorescence signal can be acquired from each voxel. The fluorescence signal $N_{a,}$ in two-photon excitation via a pulsed laser source is proportional to the number of absorbed photons per voxel and laser pulse, and is given by[1]:

$$N_a \sim \frac{P_0^2}{f^2 \tau} \left(\frac{\lambda}{A}\right)^2 A\delta \quad (1)$$

with P_0 being the average laser power at the sample plane, f the laser's pulse repetition rate, τ the pulse length, λ the central wavelength, and A the excitation area at the sample and δ the axial confinement of excitation.

Thus, the number of emitted fluorescence photons from a TeFo (T) and diffraction-limited (D) PSF for a voxel dwell time Δt are given by

$$N_{a,T} \sim \frac{P_0^2}{f_T^2 \tau} \left(\frac{\lambda}{A_T}\right)^2 A_T \delta_T \, \Delta \, t_T f_T \quad (2)$$

and

$$N_{a,D} \sim \frac{P_0^2}{f_D^2 \tau} \left(\frac{\lambda}{A_D}\right)^2 A_D \delta_D \, \Delta \, t_D f_D \, , \quad (3)$$

respectively.

Irrespective of whether a TeFo or a diffraction limited excitation is used, from the equations (B) and (C) it can be seen that

$$N_a \sim \frac{P_0^2}{f\tau} \, , \quad (4)$$

i.e. the fluorescence signal is inversely proportional to the pulse repetition rate which implies that for a given average power the signal is maximized for the lowest possible repetition rate and therefore for the maximum pulse energy. Given that each imaging voxel needs at least one pulse for excitation we

conclude that the fluorescence signal is maximized when one pulse per pixel is used.

Combining the one pulse per pixel excitation strategy with an enlarged sculpted PSF provides further advantages in the obtainable signal to noise ratio compared to the diffraction-limited excitation strategy.

Assuming the same power density (J/(μm² s)) of the excitation light and that in Eq. (1) the excitation volume of the TeFo spot was chosen to be a multiple (here denoted by V) of the diffraction limited excitation volume, i.e. $A_T\delta_T = V\times A_D\delta_D$ then it follows for the TeFo case that

$$N_{a,D}\sim\frac{P_0^2}{f_T^2\tau}\left(\frac{\lambda}{A_T}\right)^2\frac{1}{V}\times A_T\delta_T\Delta t_T f_T\ , \tag{5}$$

i.e., the number of absorbed photons per voxel and laser pulse in the case of TeFo excitation are $N_{a,T} = V\times N_{a,D}$. As a consequence, the fluorescence signal will also be higher by a factor of V when a TeFo excitation is used when the emitted fluorescence signal from a diffraction limited spot is compared to that of a TeFo spot at the same power density and dwell time.

Next we compare the fluorescence signal emitted from a TeFo spot to the sum of diffraction limited spots comprising the same volume and acquired at the same total dwell time and power density as the TeFo spot. To image the same region as in the TeFo case, the diffraction-limited focus must be scanned over the neuron (illustrated in Supplementary Fig. S1) while the recorded signal is being integrated. In order to scan V voxels at the same acquisition rate, the voxel dwell time in this case must be reduced by at least $1/V$ even when assuming no overhead due to finite scan speed. Moreover, in this scenario given that each voxel again still needs to be excited by at least one pulse, it follows that the laser repetition rate needs to be increased by V. Assuming the same average power as in the TeFo case, this leads to a decrease in pulse energy by V, which however nonlinearly reduces the obtained signal from each voxel. Thus, the overall obtained signal from the sum of all voxels (denoted by subscript MD) that comprise the same volume as the TeFo PSF is:

$$N_{a,MD}\sim V\times\frac{P_0^2}{V^2 f_T^2\tau}\left(\frac{\lambda}{A_T}\right)^2\frac{1}{V}\times A_T\delta_T\frac{1}{V}\Delta t_T V f_T\ , \tag{6}$$

which is by a factor of V^2 lower than TeFo. Thus, for the same power density, average power and dwell time, the TeFo based excitation leads to a higher obtainable signal than both the diffraction limited excitation and the sum of multiple diffraction limited spots that add up to the TeFo PSF volume.

There are several contributions to noise including shot noise, fluorescence fluctuations and electronic noise related to the data acquisition such as read-out noise. Under the assumption that the noise is dominated by shot noise, we find that the noise is identical in the two excitation schemes. This is a consequence of the Bienaymé formula which states that the variance of the sum of uncorrelated random variables equals the sum of their variances.

Since the signal is proportional to N_a, i.e. Signal$\sim N_a$, the resulting signal-to-noise ratio, SNR = Signal/$\sqrt{\text{Signal}}$ = $\sqrt{\text{Signal}}$, for TeFo excitation is given by $\text{SNR}_T = \sqrt{V}\,\text{SNR}_D$ compared to the single diffraction-limited PSF and $\text{SNR}_T = V\,\text{SNR}_{MD}$ for scanned multiple diffraction-limited excitation.

The above ratios are for an idealized case and represent a conservative estimate. Noise sources such as read-out noise, which accumulate during the acquisition process, further reduce the SNR in case of diffraction-limited excitation. In the experiment shown in this work, $V \sim 130$ taking into account the geometry of the focal spot. Therefore, as a consequence, the SNR of TeFo can be more than one order of magnitude higher compared to the diffraction-limited case.

This increase in SNR can be used to lower the power requirements compared to conventional diffraction limited 2p laser scanning microscopy, in order to reduce photo-damage and to circumvent limitations due to the saturation of the fluorophores. Alternatively, for the same power density, this gain can be used to lower the dwell time of each voxel in order to allow image acquisition at a higher speed.

Supplementary Note 2 Design considerations in s-TeFo regarding V-FOV and laser pulse repetition rate

As outlined above the imaging approach of s-TeFo is based on scanning an enlarged, sculpted PSF in a one-laser-pulse-per-image-voxel scheme. Therefore, in this approach the (volumetric) imaging FOV together with the desired spatial resolution determine the voxel rate and therefore the required laser repetition rate. Furthermore, overheads related to scanning in the lateral as well as axial directions have to be considered. In our work, we use a resonant scanner of frequency f_r for scanning in the x-direction with a lateral fill fraction t_f (i.e. the central portion of the sinusoidal resonant mirror trajectory during which signal is acquired), and use a galvo mirror for scanning the beam in the y-direction, allowing a galvo flyback time of t_g. In the axial direction, the number of planes to be scanned, n_z, and the time it takes for the piezo to return to its original position, i.e. the axial flyback time t_p, also contribute to the overall volume acquisition rate. The number of pixels to be scanned in the lateral dimensions, n_x and n_y, depend on the respective desired dimensions of the field-of-view and the lateral spatial resolution. With these parameters, the laser repetition rate, F_l, required to achieve a desired volume acquisition rate, Vps, is given by

$$F_l = \frac{n_x \cdot (n_y + 2 \cdot t_g \cdot f_r) \cdot n_z \cdot Vps}{t_f \cdot (1 - t_p \cdot Vps)} \quad (7)$$

In our work, we have chosen $n_x = n_y = 120$. Furthermore, our aim was to image a volume which extends over ~500x500x500µm. For a desired volume rate of >3Hz, this required a laser repetition rate of ~4.14MHz, given the parameters mentioned above as well as t_f =0.71, t_g = 1.1ms, n_z =51, and t_p =30ms (see Methods). Consistent with this requirement our FCPA laser provided sufficient

pulse energy (~500nJ at the output) at 4.16MHz to generate detectable GCaMP fluorescence even in deeper layers of the above volume.

Supplementary Table 1 | Comparison of state-of-the-art multi-photon calcium imaging techniques in mouse cortex with our s-TeFo approach. Methods based on one-photon excitation have not been included here due to their susceptibility to scattering, which makes them more suitable to a different range of imaging applications. Note that random access-scanning and 3D line-scan approaches do not image the entire volume and thus are currently not well-suited for imaging of awake mice. Furthermore, we note that speed is given in either frames (fps) or volumes per second (vol/s), depending on the actual type of demonstration (see reference). Volume speed performance of resonant two-photon microscopy is an extrapolated figure.

Imaging Technology	Typical Volume/2D-FOV	Resolution	Speed	Typical Depth	Strength	Limitations
Resonant two-photon scanning microscopy Review see e.g. Ref. [2]	~350x350x500µm	diffraction limited	<0.1 vol/s	<1mm	High spatial resolution, broadly disseminated and commercially available	Low frame / volume acquisition rate, ultimately limited by fluoresces lifetime
3D line-scan two-photon microscopy here: Ref [3]	~250x250x250µm	diffraction limited	10 vol/s	<250µm	Higher acquisition rate, high spatial resolution	Limited to~ 200 µm in mouse cortex, susceptible to motion
Random-access two-photon microscopy [4-6] here: Ref [5]	~400x400x500µm	diffraction limited	100 vol/s	<600µm	High neuron access speed, flexible scan trajectories	Discontinuous scanning, requires *a-priori* knowledge of location, power inefficient, sensitive to motion
Statistical multi-plane demixing here: Ref [7]	500x500µm	diffraction limited	10 fps	<500µm	Simultaneous recording from multiple planes at high speed	Limited scalability, requires sparse signal, limited laser power
Temporal multiplexing [8-10] here: Ref [11]	400x400µm	diffraction limited	250 fps	<300µm	Simultaneous recording from multiple sites/planes	No further scalability due to fluorophore lifetime, limited laser power
Scan-TeFo (this work)	~500x500x500µm	5x5x10µm	3 vol/s	<500µm	High speed and large FOV; flexible laser source; large population size	Resolution, limited to somatic activity, requires amplified laser system

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